

## **AMENDMENTS TO THE SPECIFICATION**

**Please replace the paragraph starting at line 14 on page 2 with the following amended paragraph:**

Some experiments have shown that alcohol and detergent washes during ion exchange chromatography are effective in reducing the protein associated LPS levels while poor separation of LPS from the proteins was obtained by the denaturing HIC procedure. The detergents (~~Zwittergent~~ ZWITTERGENT® 3-12 or 3-14) were shown to be more effective washing agents than the alcohols. Improved LPS clearance was also been achieved while the LPS-protein complexes were bound to a cation exchange resin as opposed to an anion exchange resin but the washing procedures used to remove LPS were effective on both matrices. When the wash procedure is performed on a cation exchanger, once the LPS-protein interactions have been disrupted the LPS should be washed out of the column while the protein is retained. During anion exchange chromatography, the LPS, being negatively charged at most pHs, remains bound to the resin along with the protein. Even though the alcohol and detergent washes were successful at reducing the levels of LPS in the LPS-protein complexes, scaling up and implementing any of these procedures in a manufacturing setting would not be practical. The concentrations of ethanol and isopropanol required to effectively reduce the LPS levels of the LPS binding proteins were greater than 50% (v/v). At these concentrations, these solutions are considered flammable liquids and as such impose many safety and operational restrictions.

**Please replace the paragraph starting at line 2 on page 4 with the following amended paragraph:**

Figure 1a is an illustration of the SP ~~Sepharose~~ SEPHAROSE® Fast Flow Elution Profile of BODIPY-LPS. The dotted line is UV signal at 280 nm and the solid line corresponds to BODIPY fluorescence expressed in relative fluorescence units (RFU).

**Please replace the paragraph starting at line 6 on page 4 with the following amended paragraph:**

Figure 1b is an illustration of the SP ~~Sepharose~~ SEPHAROSE® Fast Flow Elution Profile of BODIPY-LPS-Transferrin Complex. The dotted line is UV signal at 280 nm and the solid line corresponds to BODIPY fluorescence expressed in relative fluorescence units

(RFU).

**Please replace the paragraph starting at line 11 on page 4 with the following amended paragraph:**

Figure 2 is an illustration of BODIPY-LPS Elution Profiles of BODIPY-LPS-Transferrin Complexes on SP ~~Sepharose~~ SEPHAROSE® Fast Flow in Conjunction with Alkanediol Washes. BODIPY-LPS-transferrin complexes were generated, loaded onto a SP Sepharose Fast Flow column and the column washed with 50% solutions of 1,4-butanediol, 1,6-hexanediol, or 1,2-hexanediol.

**Please replace the paragraph starting at line 17 on page 4 with the following amended paragraph:**

Figure 3 is an illustration of the reduction of BODIPY-LPS from BODIPY-LPS-Transferrin Complexes in SP ~~Sepharose~~ SEPHAROSE® Fast Flow Eluates by Alkanediols. Zero percent reduction corresponds to a control run without an alkanediol wash. 1, 1,2-hexanediol; 2, 1% Zwittergent 3-14; 3, 1,6-hexanediol; 4, ethylene glycol; and 5, 1,4-butanediol.

**Please replace the paragraph starting at line 23 on page 4 with the following amended paragraph:**

Figure 4 is an illustration of the reduction of BODIPY-LPS from BODIPY-LPS-BSA complexes in SP ~~Sepharose~~ SEPHAROSE® Fast Flow eluates by Alkanediols. Zero percent reduction corresponds to a control run without an alkanediol wash. 1, 1,2-hexanediol; 2, 1% Zwittergent 3-14; 3, 1,2-butanediol; 4, 1,6-hexanediol; 5, 50% isopropanol; 6, 75% ethanol; 7, 1,4-butanediol; and 8, ethylene glycol.

**Please replace the paragraph starting at line 4 on page 5 with the following amended paragraph:**

Figure 5 is an illustration of viscosities of Alkanediol, Isopropanol, Ethanol, and ~~Zwittergent~~ ZWITTERGENT® Solutions in 100 mM Acetate, pH 4.5. All solutions were prepared with 100 mM Acetate buffer, pH 4.5. 1, 50% 1,6-hexanediol; 2, 50% 1,2-hexanediol; 3, 50% 1,4-butanediol; 4, 50% 1,2-butanediol; 5, 50% Ethylene glycol; 6, 50%

Isopropanol; 7, 75% Ethanol; 8, 1% ~~Zw~~ ZWITTERGENT® 3-14; and 9, 100 mM Acetate, pH 4.5.

**Please replace the paragraph starting at line 3 on page 7 with the following amended paragraph:**

In other embodiments, various processes are utilized to assist and/or facilitate separation of the protein-LPS complex. In various examples of these embodiments, the protein-LPS complex is attached to a substrate. Various methods of attachment include retaining, attracting, binding, applying, immobilizing and/or removably affixing to a substrate. Either the protein or the LPS may be attached. In an embodiment, the LPS-protein complex is bound or immobilized on a resin. Suitable resin types include, but are not limited to affinity resins, anion exchange resins, cation exchange resins, and the like, such as a SP ~~Sepharose~~ SEPHAROSE® Fast Flow resin (SPSFF resin). However, the choice of resins is a matter of routine skill in the art and can be made to serve the particular needs of the process.

**Please replace the paragraph starting at line 24 on page 10 with the following amended paragraph:**

Bovine albumin (BSA), bovine holo-transferrin, lactoferrin from bovine milk, lysozyme from chicken egg whites, lipopolysaccharides from *Escherichia coli* serotype O55:B5, and BSTFA were purchased from Sigma Chemical Co. (St. Louis, Mont.). Acetic acid, Tris (base), sodium hydroxide (NaOH), hydrochloric acid, sodium chloride (NaCl), ethanol, isopropanol, sodium dodecyl sulfate (SDS), and sodium phosphate dibasic 7-hydrate were purchased from J.T. Baker Chemical Co. (Phillipsburg, N.J.). 1,6-Hexanediol was from BASF Co. (Mount Olive, N.J.). 1,2-Hexanediol, 1,2-butanediol, and Zwittergent 3-14 (~~Zw~~ 3-14) were purchased from Fluka (Milwaukee, Wis.). 1,4-Butanediol and ethylene glycol were purchased from Aldrich (Milwaukee, Wis.). Phosphate buffered saline (PBS), 10x, was purchased from Bio-Rad Laboratories, Inc. (Hercules, Calif.). *Escherichia coli* BODIPY® FL conjugate lipopolysaccharide, serotype O55:B5, (BODIPY-LPS) and EnzChek Lysozyme Assay Kit were purchased from Molecular Probes, Inc. (Eugene, Oreg.). Pyrosol, *Limulus Amebocyte* Lysate (LAL) ~~Pyrotell-T~~ PYROTELL-T, LAL reagent water (LRW), and control standard endotoxin from *E. coli* O113:H10 (CSE), were obtained from Associates of Cape Cod, Inc. (Falmouth, Mass.). SP ~~Sepharose~~ SEPHAROSE® fast flow (SPFF) resin, Q ~~Sepharose~~ SEPHAROSE® fast flow (QFF) resin, and HR 10/10 columns were from

Amersham Biosciences (Piscataway, N.J.). Clear polystyrene 96-well microtiter plates were from Associates of Cape Cod, Inc. (Falmouth, Mass.) and black 96-well microtiter plates from NUNC (Rochester, N.Y.).  $\beta$ -Hydroxytetradecanoic acid,  $\beta$ -hydroxytridecanoic acid,  $\beta$ -hydroxyundecanoic acid,  $\beta$ -hydroxytridecanoate,  $\beta$ -hydroxytetradecanoate, and  $\beta$ -hydroxyundecanoate were purchased from Matreya, Inc. (Pleasant Gap, Pa.). Heptane was purchased from Spectrum (New Brunswick, N.J.).

**Please replace the paragraph starting at line 6 on page 13 with the following amended paragraph:**

Samples were adjusted to a pH between 6 and 8 with ~~Pyroset~~ PYROSOL, if needed. CSE and ~~Pyrotell-T~~ PYROTELL-T were reconstituted with LRW. The linear curve of CSE was from 0.03 to 1.00 EU/ml. The analytical procedure of LAL KTA was as follows. To each well of a clear polystyrene microtiter plate 100  $\mu$ l of sample or standard and 100  $\mu$ l of ~~Pyrotell-T~~ PYROTELL-T were added. For spiked samples, 5  $\mu$ l of 2.00 EU/ml CSE was added to obtain 0.10 EU/ml CSE level. The plate was shaken for 10 seconds and data collected, every minute, in the kinetic mode at 405 nm for 1 hour at 37°C.

**Please replace the paragraph starting at line 12 on page 15 with the following amended paragraph:**

For transferrin, a SP ~~Sepharose~~ SEPHAROSE® Fast Flow column was charged with 100 mM Acetate, 1 M NaCl, pH 5, and equilibrated with 100 mM Acetate, pH 5. After loading, the resin was washed with the equilibration buffer and then eluted with 50 mM sodium phosphate, 1 M NaCl, pH 7.5. When an organic or detergent wash was performed, it was applied after the initial wash step and was for 6 CV unless otherwise stated. This wash was followed by a second wash with equilibration buffer to remove the organic or detergent prior to elution.

**Please replace the paragraph starting at line 7 on page 16 with the following amended paragraph:**

For BSA, a Q ~~Sepharose~~ SEPHAROSE® Fast Flow column was charged with 50 mM Tris, 1 M NaCl, pH 8.0 and equilibrated with 50 mM Tris, pH 8.0. After loading, the resin was washed with equilibration buffer. BSA was eluted with 25 mM Acetate, pH 4.5, and LPS with 25 mM Acetate, 1 M NaCl, pH 4.5. When an alkanediol wash was performed, it was

inserted after the initial wash step and was for 6 CV. This wash was followed by a second wash with equilibration buffer to remove the alkanediol.

**Please replace the paragraph starting at line 20 on page 17 with the following amended paragraph:**

SP ~~Sepharose~~ SEPHAROSE® Fast Flow Chromatography of LPS and LPS-Protein Complexes

The LPS elution profiles of LPS by itself and LPS-BSA complexes on SP ~~Sepharose~~ SEPHAROSE® Fast Flow resin were determined by LAL-KTA analysis of selected column fractions (Table III). When LPS was chromatographed by itself the LPS was detected primarily in the wash-unbound fraction as expected. Chromatography of the LPS-BSA complexes resulted in the majority of the LPS being detected in the BSA eluate fraction confirming the LPS binding property of BSA (Dziarski, 1994) and demonstrating that the BSA-LPS complexes are stable under cation exchange chromatography conditions employed.

**Please replace title for TABLE III on page 18 with the following amended title for TABLE III:**

**TABLE III SPFF Chromatography Elution Profiles of LPS and LPS-BSA**

LPS and LPS-BSA complex were chromatographed on SP ~~Sepharose~~ SEPHAROSE® Fast Flow. Column fractions were analyzed for LPS by the LAL KTA as described in the methods.

**Please replace title for TABLE IV on page 19 with the following amended title for TABLE IV:**

**TABLE IV SPFF Chromatography Elution Profiles of BODIPY-LPS and BODIPY-LPS-Protein**

BODIPY-LPS and BODIPY-LPS-protein complex were chromatographed on SP ~~Sepharose~~ SEPHAROSE® Fast Flow. Column fractions were analyzed for BODIPY-LPS by the BODIPY assay as described in the methods.

**Please replace the paragraph starting at line 1 on page 20 with the following amended paragraph:**

The effectiveness of a series of alkanediols to remove LPS from proteins while the proteins were bound to ionic solid supports were compared to those of ethanol, isopropanol, and ~~Zwittergent~~ ZWITTERGENT® 3-14, which had been shown to be effective in removing LPS from a LPS binding protein. A SP ~~Sepharose~~ SEPHAROSE® Fast Flow column was loaded with BODIPY-LPS-transferrin complex. The resin was washed with six column volumes of a 50% alkanediol solution and then eluted. Fractions were collected and assayed for BODIPY-LPS (Figure 2). As the chain length of the alkanediol was increased from four to six carbons, the fluorescence of the alkanediol wash fractions increased while the fluorescence of the eluate fractions decreased. This demonstrated that alkanediols removed BODIPY-LPS from the transferrin complex. Figures 3 and 4 illustrate the effects of alkanediol structure on BODIPY-LPS removal from transferrin and BSA, respectively. BODIPY-LPS removal efficiency increased with increasing alkanediol chain length and the 1,2-alkanediol isomers were more effective than the terminal alkanediols at removing the BODIPY-LPS. 1,2-hexanediol was the most efficient compound tested and out performed the detergent and alcohols. 1,2-butanediol and 1,6-hexanediol as well as 50% isopropanol and 75% ethanol reduced the BODIPY-LPS associated with transferrin to similar levels. The removal of BODIPY-LPS by the alkanediols was similar for both the transferrin and BSA complexes.

**Please replace the paragraph starting at line 21 on page 20 with the following amended paragraph:**

Reference to Figure 2 illustrates BODIPY-LPS Elution Profiles of BODIPY-LPS-Transferrin Complexes on SP ~~Sepharose~~ SEPHAROSE® Fast Flow in Conjunction with Alkanediol Washes.

**Please replace the paragraph starting at line 1 on page 21 with the following amended paragraph:**

Figure 3 illustrates the Reduction of BODIPY-LPS from BODIPY-LPS Transferrin Complexes in SP ~~Sepharose~~ SEPHAROSE® Fast Flow Eluates by Alkanediols. Chromatography was as described in Figure 2. Zero percent reduction corresponds to a control run without an alkanediol wash. 1, 1,2-hexanediol; 2, 1% ~~Zwittergent~~

ZWITTERGENT® 3-14; 3, 1,6-hexanediol; 4, ethylene glycol; and 5, 1,4-butanediol. BODIPY-LPS-transferrin complexes were generated, loaded onto a SP ~~Sepharose~~ SEPHAROSE® Fast Flow column and the resin washed with 50% solutions of 1,4-butanediol, 1,6-hexanediol, or 1,2-hexanediol. Following a wash to remove the alkanediol, transferrin was eluted as described in the methods. Sanitization between runs was with 0.5 N NaOH.

**Please replace the paragraph starting at line 11 on page 21 with the following amended paragraph:**

Figure 4 illustrates The reduction of BODIPY-LPS from BODIPY-LPS-BSA complexes in SP ~~Sepharose~~ SEPHAROSE® Fast Flow eluates by alkanediols. Chromatography was as described in FIG. 2. Zero percent reduction corresponds to a control run without an alkanediol wash. 1, 1,2-hexanediol; 2, 1% Zwittergent 3-14; 3, 1,2-butanediol; 4, 1,6-hexanediol; 5, 50% isopropanol; 6, 75% ethanol; 7, 1,4-butanediol; and 8, ethylene glycol.

**Please replace the paragraph starting at line 7 on page 22 with the following amended paragraph:**

Since 1,2-hexanediol was the most effective compound tested for removing BODIPY-LPS from both transferrin and BSA, the concentration dependence of the 1,2-hexanediol wash needed to affect this removal was investigated. The reduction of BODIPY-LPS in the SP ~~Sepharose~~ SEPHAROSE® Fast Flow BSA eluate fraction was determined after 1,2-hexanediol washes containing 5%, 20%, and 50% 1,2-hexanediol. The 5% 1,2-hexanediol wash resulted in about a 55% decrease in the BSA associated BODIPY-LPS while the reduction of BODIPY-LPS by the 20% and 50% 1,2-hexanediol washes were comparable at approximately 96%.

**Please replace title for TABLE V on page 22 and 23 with the following amended title for TABLE V:**

**TABLE V SPFF Chromatography Elution Profiles of BODIPY-LPS and BODIPY-LPS-Lactoferrin Complexes**

BODIPY-LPS-lactoferrin complexes were generated, loaded onto a SP ~~Sepharose~~ SEPHAROSE® Fast Flow column. For runs that included a 50% solution of 1,6-hexanediol,

a wash to remove the 1,6-hexanediol was included prior to elution of lactoferrin as described in the methods. Column fractions were assayed for BODIPY and 3-OH-14:0 fatty acids as described in the methods.

**Please replace the paragraph starting at line 18 on page 23 with the following amended paragraph:**

During the chromatographic runs, a rise in the system back pressure was noted when the alkanediol washes were applied. The viscosity of each organic solutions and the ~~Zwittergent~~ ZWITTERGENT® solution, prepared in 100 mM Acetate, pH 4.5, were measured (Figure 5). The viscosity of the alkanediols increased with carbon chain length while the viscosity of the 1,2-alkanediol isomers were slightly less than the terminal alkanediol isomers. The increased viscosity of the alkanediol solutions may present some difficulties in scale-up. Column flow rates may have to be adjusted to maintain suitable system pressure for the equipment in use. Being able to use lower concentrations of the alkanediols to remove LPS from the protein-LPS complexes would partially alleviate this problem. For example, 20% and 50% 1,2-hexanediol washes effectively reduce the BODIPY-LPS to approximately the same levels for BSA complexes as indicated above. The viscosity of 20% 1,2-hexanediol is about one third that of 50% hexanediol, 2.6 Cp compared to 7.5 Cp.

**Please replace the paragraph starting at line 4 on page 24 with the following amended paragraph:**

Reduction of LPS from LPS-Protein Complexes by Alkanediols during Q ~~Sepharose~~ SEPHAROSE® Fast Flow Chromatography

**Please replace the paragraph starting at line 14 on page 24 with the following amended paragraph:**

The ability of 1,6-hexanediol and 1,2-hexanediol to reduce the BODIPY-LPS levels of BSA-LPS complexes during anion exchange chromatography on Q ~~Sepharose~~ SEPHAROSE® Fast Flow resin was investigated. The two isomers, 1,2- and 1,6-, of hexanediol were chosen since these were the most effective compounds at reducing the levels of BODIPY-LPS from protein complexes during cation exchange chromatography.



**Please replace title for TABLE VI on page 25 with the following amended title for  
TABLE VI:**

**TABLE VI The QFF Elution Profiles of BODIPY-LPS and BODIPY-LPS-BSA  
Complexes.**

BODIPY-LPS and BODIPY-LPS-BSA complex were chromatographed on Q  
~~Sepharose~~ SEPHAROSE® Fast Flow. Column fractions were analyzed for BODIPY-LPS by  
the BODIPY assay as described in the methods.

**Please replace the paragraph starting at line 24 on page 25 with the following amended  
paragraph:**

Lysozyme was used to determine the effect of the washing agents on enzymatic  
activity and thereby, indirectly the denaturing effects of the washing agents during SP  
~~Sepharose~~ SEPHAROSE® Fast Flow chromatography. Lysozyme was chromatographed  
with and without a 6 CV %50 1,6-hexanediol wash or 1,2-hexanediol wash and the column  
loads and eluates assayed for lysozyme activity using a fluorescence microplate lysozyme  
activity assay. The hexanediol washes had no detrimental effects on lysozyme activity. 75%  
ethanol, 50% isopropanol, and 1% ~~Zwittergent~~ ZWITTERGENT® 3-14 washes also had no  
effect on lysozyme activity.